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Simvastatin and oseltamivir combination therapy does not improve the effectiveness of oseltamivir alone following highly pathogenic avian H5N1 influenza virus infection in mice

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ABSTRACT

Nonspecific anti-inflammatory drugs have been purported to reduce the burden of severe influenza disease. We demonstrate that, unlike oseltamivir administration, simvastatin administration did not reduce morbidity, mortality, or viral load of mice infected with H1N1 or H5N1 viruses. No added benefit to the efficacy of oseltamivir therapy was observed when mice were treated in combination with simvastatin. Modest reductions in lung cytokine production in H5N1 but not H1N1 virus-infected simvastatin-treated mice indicate a potential benefit for statin use in mitigating disease following severe virus infection.

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Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are a class of cardioprotective medications which possess pleiotropic immunomodulatory and anti-inflammatory properties (Jain and Ridker, 2005; Kwak et al., 2000). The ability of statins to inhibit cytokine production, improve endothelial cell function, and modulate host molecular pathways has led to the hypothesis that statin use could be a preventative therapy against cellular damage caused by influenza virus infection (Blanc et al., 2011; Fedson, 2006). As statins represent one of the most widely prescribed classes of drug in the world, identifying the capacity of statins to reduce influenza virus morbidity and mortality in the event of a pandemic or simply to extend the time between onset of illness and development of severe disease, thus allowing a greater window for the efficacy of existing antiviral treatments, is a matter of great public health importance.

Despite conflicting reports, several retrospective observational studies have identified an association between statin use and reductions in influenza virus morbidity in humans; however, these studies are limited by the substantial variability in timing and duration of drug administration among participants, or the inclusion of pneumonia morbidity in the absence of laboratory-confirmed influenza virus infection (Brett et al., 2011; Frost et al., 2007; Kwong et al., 2009; Mortensen et al., 2005; Vandermeer et al., 2012). Selected animal studies have further suggested a protective role for statins and other nonspecific anti-inflammatory drug regimens against acute lung injury, but results have been mixed (Budd et al., 2007; Ferraro et al., 2011; Gower and Graham, 2001; Jacobson et al., 2005; Salomon et al., 2007; Walsh et al., 2011). Recent work has examined the efficacy of statin treatments following influenza virus infection in mice, finding only limited benefits of statin treatment in the amelioration of influenza virus infection, however these studies have not generally employed contemporary, wild-type viruses and have not extensively examined statin drugs administered concurrently with commercially available antiviral treatments (An et al., 2011; Kumaki et al., 2012; Radigan et al., 2012). Furthermore, inconsistencies in statin drug administration, inoculation routes, and a paucity of studies including influenza-specific antivirals as appropriate controls among these previously published studies makes it difficult to thoroughly assess the efficacy of statin administration in the context of influenza virus infection. There is a need to investigate more fully the ability of statin drugs, alone or in combination with existing antiviral therapeutics, to mitigate influenza virus disease severity against both mild and virulent influenza virus strains. To address this question, we utilized simvastatin, a commonly prescribed statin drug, and oseltamivir,

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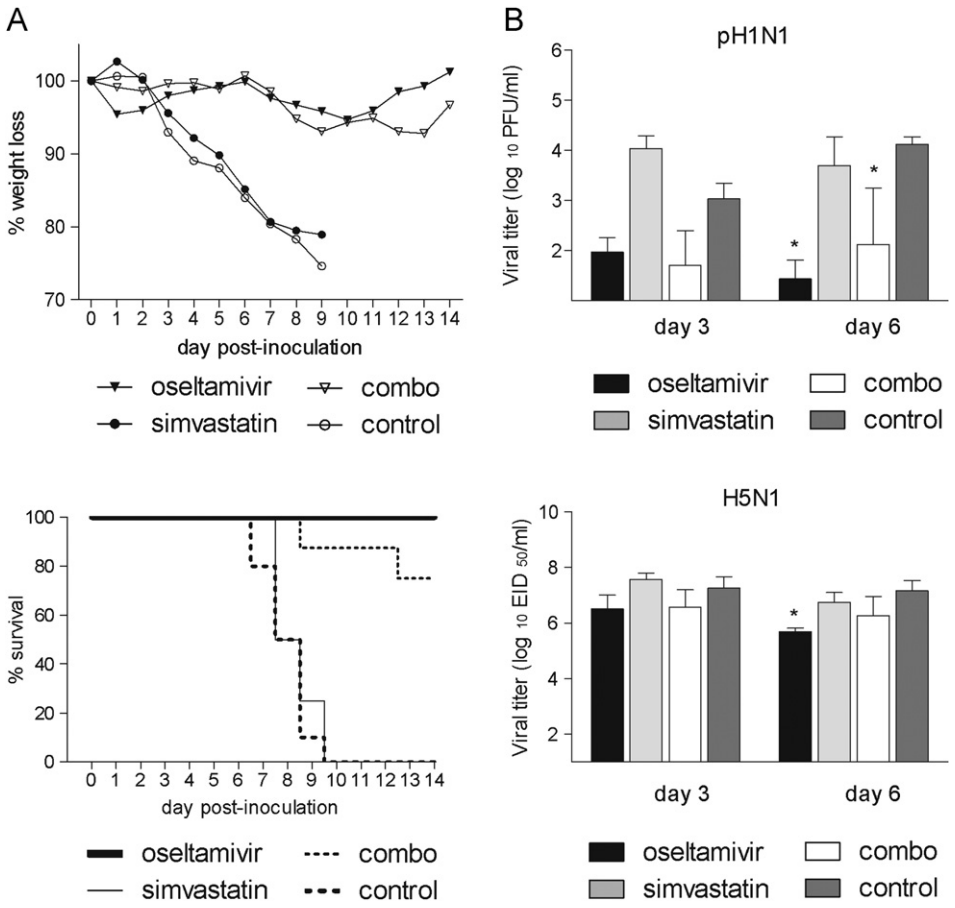

Fig. 1. Effect of statin treatment on influenza virus disease outcome and replication in mice. Oseltamivir (50 mg/kg), simvastatin (10 mg/kg), or a combination of both was administered once daily by oral gavage; control mice received vehicle only. (A) Groups of 6–10 mice were inoculated i.n. with 100 MID_{50} of H5N1 virus and monitored daily for morbidity and mortality. Any mouse which lost $>25\%$ initial body weight was euthanized. (B) Groups of 3–5 mice were inoculated i.n. with 100 MID_{50} of pH1N1 or H5N1 virus and lungs were collected days 3 and 6 p.i. for virus titration. Tissues were titrated in cells (pH1N1) or eggs (H5N1) with titers reported as plaque forming units (PFU) or 50% egg infectious doses (EID_{50})/ml of tissue, respectively. ∗, $p<0.05$ compared to control mice by one-way ANOVA with a Bonferroni post-test.

Table 1
Kinetic analysis of circulating lymphocytes following H5N1 virus challenge in mice.

	WBC[b]	Percentage of circulating lymphocytes in peripheral blood[a]				
		LY	NE	MO	EO	BA
Pre-infection	2.0 ± 0.4	76.2	16.7	6.2	0.05	0.3
Day 3						
Oseltamivir	2.2 ± 0.3	75.6*	20.1*	3.3	0.8	0.2
Simvastatin	1.4 ± 0.5	58.8	33.1	5.7	1.9	0.5
Combo	2.6 ± 1.3	70.1	25.7	3.4	0.8	0.1
Control	1.2 ± 0.3	61.8	31.1	5.9	0.7	0.5
Day 6						
Oseltamivir	3.6 ± 1.3*	70.2*	24.0*	5.2	0.5	0.1
Simvastatin	1.1 ± 0.5	58.9	34.2	3.8	2.4	0.7
Combo	2.2 ± 1.6	66.9	31.2	1.2	0.6	0.1
Control	1.0 ± 0.4	49.8	41.6	5.6	2.5	0.6

[a] Average percentage of lymphocytes (LY), neutrophils (NE), monocytes (MO), eosinophils (EO), and basophils (BA) in whole blood.
[b] WBC, total white blood cells in whole blood ± standard deviation (K/μl).
* $p<0.05$ compared with control group by one-way ANOVA with a Bonferroni post-test.

the most widely prescribed antiviral drug for influenza viruses (Gubareva et al., 2000; Jain and Ridker, 2005).

To determine the susceptibility of influenza viruses to statin treatment in vivo, we inoculated six-to-eight week old female BALB/c mice intranasally (i.n.) with two viruses which exhibit divergent phenotypes in this model. A/Chicken/Korea/Gimje/08 (H5N1) replicates efficiently in the murine respiratory tract, causing severe morbidity and mortality (D. Wadford and T.M. Tumpey, unpublished data). A/Mexico/4482/09 (A/H1N1pdm09, pH1N1) virus, isolated from the 2009 pandemic, does not cause a lethal infection (Belser et al., 2010). Simvastatin sodium salt (10 mg/kg body weight; Calbiochem) was administered by oral gavage once daily for 12 days commencing 72 h before virus inoculation (Gower and Graham, 2001; Liu et al., 2009). Oseltamivir (50 mg/kg body weight; Roche) was administered by oral gavage once daily for 8 days commencing 24 hours before virus

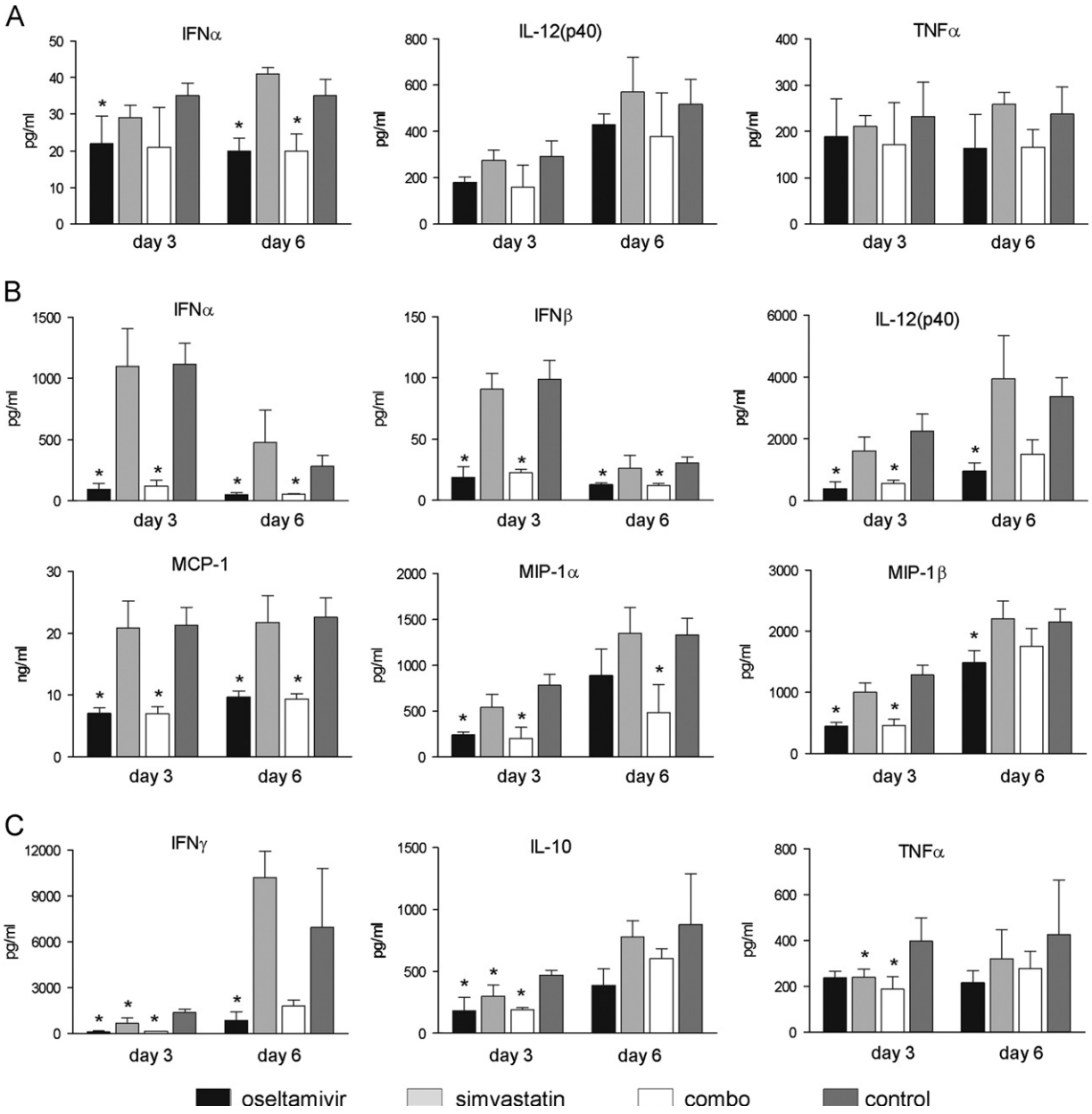

Fig. 2. Effect of statin treatment on proinflammatory cytokine and chemokine production in the lungs of mice following influenza virus infection. Oseltamivir (50 mg/kg), simvastatin (10 mg/kg), or a combination of both was administered once daily by oral gavage; control mice received vehicle only. Groups of 3–5 mice were inoculated i.n. with 100 MID_{50} of pH1N1 or H5N1 virus and lungs were collected days 3 and 6 p.i. Clarified lung homogenates were tested via BioPlex (Bio-Rad) or ELISA (R&D Systems) assays. (A) Cytokine and chemokines detected following pH1N1 virus infection. (B) Cytokines and chemokines that were not significantly altered by statin treatment following H5N1 virus infection. (C) Cytokines and chemokines following H5N1 virus infection where significant reductions in statin-treated mice compared with control mice were detected. ∗, $p < 0.05$ compared to control mice by one-way ANOVA with a Bonferroni post-test.

inoculation (Tumpey et al., 2002). While these doses are above concentrations typically administered to humans, they are necessary to achieve bioactive doses in rodents and are consistent with previously published in vivo studies (Ilyushina et al., 2008; Leung et al., 2003). Control mice received distilled water on the same schedule. Mice were inoculated with 100 mouse infectious dose 50% (MID_{50}) of each virus (equivalent to a 2 lethal dose 50% (LD_{50}) of H5N1), and 6–10 mice/group were monitored daily for 2 weeks for morbidity (measured by weight loss) and mortality. An additional three to five mice/group were euthanized on days 3 and 6 post-inoculation (p.i.) for collection of whole blood for analysis of circulating lymphocytes, and lung tissues for virus titration and analysis of proinflammatory cytokines and chemokines (Belser et al., 2010). Days 3 and 6 p.i. were chosen as they represent times of peak viral replication in this model and are established timepoints for assessment of these parameters in murine pathogenesis and antiviral studies (Belser et al., 2010; Kumaki et al., 2012; Maines et al., 2005; Wong et al., 2011).

Mice receiving daily statin administration exhibited comparable severe morbidity and 100% mortality following a lethal H5N1 virus challenge as untreated control mice (Fig. 1A). In contrast, oseltamivir treatment protected mice from weight loss and death following H5N1 virus challenge, and resulted in significantly decreased viral titers in the lung day 6 p.i. ($p < 0.05$) (Fig. 1A and B). The reductions in H5N1 viral load following oseltamivir treatment are comparable with prior studies demonstrating a modest decrease in lung virus titer at these times post-infection

(Govorkova et al., 2009; Ilyushina et al., 2007). Weight loss following pH1N1 virus challenge was not observed in any group tested ($<5\%$ of initial body weight loss in any group p.i.). However, reductions in viral load in the lung were detected following daily oseltamivir but not simvastatin administration in these mice (Fig. 1B). Mice receiving a combination of both simvastatin and oseltamivir did not exhibit an improvement in disease outcome compared with oseltamivir treatment alone following infection with either virus subtype. These results are in agreement with previous studies which found no improvement in morbidity or mortality following wild-type H5N1 virus infection in mice treated with immune modulators (Kumaki et al., 2012; Salomon et al., 2007).

H5N1 virus infection of mice can cause pronounced leukopenia and lymphopenia, but pretreatment with oseltamivir was found to protect mice from these hematopoietic alterations (Table 1) (Maines et al., 2005). In contrast, simvastatin did not protect against leukopenia following H5N1 virus infection and did not significantly reduce the degree of lymphocyte depletion compared with control mice (Table 1). Combination therapy did not confer additional benefit compared to the respective monotherapies. While simvastatin pretreatment of mice contributed to maintaining a normal balance of immune cell subpopulations following non-virus induced acute lung injury in a prior study, our findings are in accord with a recent study which detected comparable levels of total leukocyte counts in bronchoalveolar lavage fluid between statin-treated and untreated control mice following influenza virus infection (Ferraro et al., 2011; Radigan et al., 2012).

Previous mouse studies have found that oseltamivir treatment could reduce lung inflammatory responses induced by influenza virus infection (Ilyushina et al., 2008; Wong et al., 2011). Similarly, we found that oseltamivir treatment resulted in the significant reduction of numerous proinflammatory cytokines and chemokines associated with influenza virus infection, predominantly following lethal H5N1 virus infection (Fig. 2A, B, and data not shown) (Maines et al., 2008). Interestingly, simvastatin treatment significantly reduced ($p<0.05$) the production of IFNγ, IL-10, and TNFα in the lungs of H5N1 virus-infected mice on day 3 p.i.; all three cytokines are known targets of statin drugs with roles in the initiation of inflammatory cell infiltration (Fig. 2C) (Jain and Ridker, 2005). Notably, the observed decreased production of TNFα in statin-treated mice is in agreement with previous work which has identified a role for TNFα in influenza virus morbidity, with reduced production of TNFα in mice receiving statins following lung injury in mice (Ferraro et al., 2011; Szretter et al., 2007). While we observed significant reductions of selected cytokines following statin treatment in H5N1 virus-infected murine lungs, these differences were not observed following pH1N1 virus-infection (Fig. 2A and data not shown), in agreement with Radigan et al. who did not observe differences in cytokine levels in rosuvastatin-treated mice following H3N2 or H1N1 virus infection (Radigan et al., 2012). No significant differences in cytokine production were detected between mice receiving oseltamivir alone and those receiving combination therapy (Fig. 2). Treatment with simvastatin in the absence of virus infection did not result in substantial alterations of proinflammatory cytokines and chemokines compared with uninfected control mice (data not shown).

Host innate immune responses contribute to the severity of disease induced by H5N1 viruses, and depletion of cytokines and/or chemokines can influence the resulting course of disease (Maines et al., 2008; Szretter et al., 2007). The findings in this report support previous studies which demonstrate limited improvements in cellular infiltration and reduced cytokine production in the lungs of mice following administration of immunomodulatory drugs in the absence of improved survival following lung injury caused by viral, bacterial, and other stimuli (Boyd et al., 2012; Ferraro et al., 2011; Walsh et al., 2011). While we did not observe a striking antiviral effect of simvastatin administration alone following influenza virus infection, or a measurable improvement of combination therapy over oseltamivir treatment alone in mice, reduced hypercytokinemia following H5N1 but not pH1N1 virus infection in mice following simvastatin administration supports a potential benefit of this treatment depending on the virus used to infect, warranting further study of statin administration in the mitigation of severe influenza disease. As shown in animal studies, reductions in influenza virus-induced pulmonary inflammation by statin administration may be of particular benefit in severe cases of influenza virus infection where mechanical ventilation is employed (Muller et al., 2010).

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